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Symposium on the Fine Structure and Replication of Bacteria and Their Parts

IV. Unbalanced Cell-Wall Synthesis: Autolysis and Cell-Wall Thickening[1]

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INTRODUCTION

At least two types of cell-wall replication have been shown to occur in growing bacteria by use of the immunofluorescent technique (6, 7, 8, 9, 10, 28). With some bacteria, such as *Streptococcus pyogenes*, wall synthesis appears to occur at specific sites, rings or bands, on the circumference of the cell (8, 10). These seem to be the sites at which the next cross-wall formation will occur. In this situation, new wall can be distinguished from old by immunofluorescence. With other bacteria, such as *Salmonella*, wall growth seems to be diffuse over the entire cell surface (8, 9, 28).

During balanced growth, a greater surface area is normally required to cover a greater volume of protoplasm. A cell wall covers the entire surface of the bacterial cell. The cell walls of a wide variety of bacterial species appear to be rigid, rather insoluble, protective, continuous structures of complex and somewhat unique composition (40, 42, 44). A small amount of damage to the wall usually results in osmotic fragility or cell lysis (26, 31). It is difficult to envision a mechanism for increasing the surface area of the bacterial cell wall that does not involve structural weakness at the areas of active wall synthesis.

This is especially true for those species in which a ring or band of new wall synthesis has been seen (6, 7, 8, 10). A simple analogy can be visualized by attempting to add more beads to a circular string of beads, thereby increasing the size of the circle, without breaking or opening the string. In this connection it is interesting to note that Cole and Hahn (10) observed in *S. pyogenes* that a second, new band of wall synthesis was initiated about midway between the previous band and the end of the coccus. Wall synthesis was initiated at the second band before cell division took place at the previous band. Therefore, for this discussion, I shall assume that there are areas or points of incipient weakness in the growing wall structure. To simplify the discussion, and since our work has been with a streptococcus (*S. faecalis* ATCC 9790), only the *S. pyogenes* type of wall replication will be considered. There is probably not a real difference in the *S. typhosa* type of situation except, perhaps, that it may be more complicated. It is of interest to note that Chung et al. (7) investigated cell-wall replication in a strain of *S. faecalis* by use of a fluorescent-antibody technique. They found that new wall synthesis occurred at localized bands at the coccal equator. Their results are similar to those of Cole and Hahn (10) with *S. pyogenes*, except that only one band of wall synthesis per coccus was seen.

Cole and co-workers examined cell-wall replication in what is commonly called a "balanced" growing situation. The bacteria are in a nutritionally adequate medium that permits continued growth and division in a constant and regular manner, the steady-state situation which is commonly known as exponential growth. During this process, nearly all of the cells in the culture, more or less regularly, increase in size and then divide. In a continuous-culture apparatus, or by other means of constantly transferring cells to a fresh, identical environment, this steady state may continue indefinitely. Therefore, during an exponential growth phase, all of the cellular constituents, the macromolecules, and structures such as the cellular deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, ribosomes, membranous structures, and walls, must

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be synthesized in a controlled, regular, and balanced fashion for the conditions used.

"Unbalanced growth" can occur. The term "unbalanced growth" will be used to describe conditions where cellular growth and division no longer occur in a steady state. The unbalanced situations to be described will result from the addition or omission of known and controllable single environmental factors to or from a balanced exponentially growing culture which is at its maximal growth rate for the conditions employed. The only change is the addition or omission of a single factor to the otherwise complete medium. All of the factors we have studied thus far which cause unbalanced growth seem to result in biochemical and morphological changes. These changes are reflected in a pattern of favored synthesis of specific cellular macromolecules and structures. The pattern of these changes seems to be at least quantitatively different for each individual factor.

All of our studies have been with *S. faecalis* (ATCC 9790) grown in a previously described highly buffered complete synthetic medium (47). This medium is by no means a minimal medium. In addition to a large number of nutritionally indispensable vitamins and amino acids, a variety of "nonessential" substances are included. Most of the "nonessential" substances do not appear to be completely dispensable in terms of either maximal growth rate or maximal growth level (61). In the complete medium, which contains 0.3 M phosphate buffer, exponential growth, at a mass doubling time of 31 to 33 min, can occur to cell densities greater than 1 g (dry weight) per liter. Maximal cell densities of over 2 g per liter have been obtained in this medium (51).

Most recently our studies have involved two types of unbalanced growth. These two types are based on the presence (Fig. 1A) or absence (Fig. 1B) of continued cell-wall synthesis. Balanced exponential growth and division occur when the continued synthesis of new cell-wall polymers is accompanied by the balanced synthesis of protein and other cellular macromolecules (Fig. 1A-1). However, inhibition of protein synthesis with the continued synthesis of cell-wall polymers can result in cells with thickened cell walls (Fig. 1A-2). Protein synthesis may be selectively inhibited by use of chloramphenicol. Deprivation of a nutritionally essential amino acid that is not a major component of the cell wall, such as valine or threonine, also is effective as an inhibitor of protein synthesis.

On the other hand, prevention of the continued synthesis of new cell-wall polymers can result in osmotic fragility (Fig. 1B). This can be done by

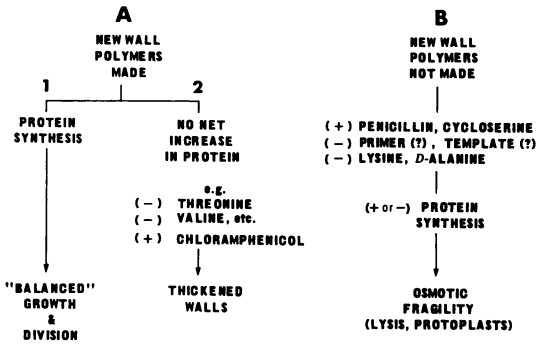

FIG. 1. *Schematic representation of balanced and unbalanced growth as governed by the synthesis of cell-wall substances.*

addition of an inhibitor of cell-wall synthesis, such as penicillin (25, 39) or cycloserine (46, 56), without significant inhibition of the synthesis of proteins or other cellular macromolecules. The absence of an effect of either of these antibiotics on cellular processes other than cell-wall synthesis has been well documented. For example, there have been numerous demonstrations (for reviews, see 26 and 31) that spherical, osmotically fragile forms which contain little or no cell wall can increase in size and mass in the presence of penicillin. In addition, both penicillin and cycloserine, at concentrations over eight times those required to inhibit growth of approximately the same number of streptococci, showed no effect on the increase in mass of lysozyme-produced protoplasts of *S. faecalis* (52). Under the same experimental conditions, 14 other antibacterial agents were effective inhibitors of protoplast "growth" in the range of concentration that was effective against the streptococci. Inhibition of the synthesis of new wall polymers also can be accomplished by means of deprivation of a nutritionally essential substance. This substance can be either a major component of the cell wall or one that is catalytically involved in the synthesis of such a major wall component. In some cases, deprivation of a single amino acid, which is not a major component of cellular proteins and which is found essentially exclusively in the cell wall, results in osmotic fragility and lysis under conditions that appear to allow continued synthesis of protein and other cellular macromolecules. Examples of this are the deprivation of diaminopimelic acid in certain *Escherichia coli* mutants (2, 30, 32, 41) and of D-alanine in *S. faecalis* (49). McQuillen (29) compared conditions of metabolic disturbance that result in osmotic fragility (such as diaminopimelic acid deprivation in *E. coli* mutants and

the production of spherical, osmotically fragile bodies by means of growth in the presence of penicillin) with conditions that do not seem to result in osmotic fragility. From this comparison, McQuillen (29) concluded that lysis can result from the failure of cell-wall growth to keep up with the active processes of continued growth of cytoplasm.

However, production of osmotic fragility seems to be independent of the occurrence of continued synthesis of protein or other cellular macromolecules. Osmotic fragility can be obtained in *S. faecalis* when continued protein synthesis also is inhibited. For example, osmotic fragility can result from the withdrawal of the nutritionally essential amino acid lysine. Lysine is not only a component of the cell-wall mucopeptide but is also a component of cytoplasmic proteins (51). The absence of continued synthesis of new cell-wall substance seems to be the deciding factor in producing osmotic fragility. The occurrence or inhibition of protein synthesis seems to be only incidental. An interpretation of these findings is discussed below.

Another factor which could possibly lead to osmotic fragility is indicated in Fig. 1 with a question mark, since it has not yet been thoroughly investigated. This is the role of primers and templates in the synthesis of the cell-wall heteropolymers (40, 42). Primers or templates, or both, have been found to play an important role in the biosynthesis of a number of biologically important heteropolymers, including proteins, nucleic acids, and at least several heteropolysaccharides. There are a number of examples of protoplasts, spheroplasts, and L-forms that seem to be unable to synthesize cell-wall polymers (26, 31). For example, Edwards and Panos (12) demonstrated the presence of uridine diphosphate muramic acid precursors of cell-wall mucopeptide in a stable, penicillin-induced L-form of a group A streptococcus. A similar nucleotide containing muramic acid peptide was not demonstrated in the parent coccus. However, attempts to demonstrate the presence of the rigid cell wall, wall mucopeptide, or rhamnose-containing wall polysaccharide in this same L-form were all negative (38). As yet, we do not know why the streptococcal L-form fails to synthesize a cell wall. Our limited knowledge leaves open a number of possibilities: (i) the absence of a specific wall-polymerizing enzyme, such as that described by Chatterjee and Park (5) for the synthesis of wall mucopeptide; (ii) the absence of a wall-hydrolyzing enzyme required to open bonds for the insertion of new material, such as the autolytic system of *S. faecalis* (49, 51, 54, 55); and (iii) the L-form may lack the specific structural sites that may be involved in wall polymerization. Fitz-James (13), with *Bacillus megaterium*, and Ryter and Landman (43), with *B. subtilis*, have presented evidence indicating that protoplasts and L-forms have lost the membranous mesosome structures. Ryter and Landman (43) have postulated a causal relationship between absence of mesosomes and inability to synthesize the cell wall. Lastly, it is perhaps at least equally possible that the absence of a rigid cell wall in L-forms may result from the absence of a primer or a template, or both, at the site of new cell-wall synthesis.

UNBALANCED GROWTH RESULTING IN OSMOTIC FRAGILITY

Conditions needed to obtain autolysis in *S. faecalis* 9790 cultures have been studied in some detail (49, 54), and the results suggest that the autolytic process has two distinct aspects. First, there are conditions that lead to the formation of cells that are prone to autolysis, and, second, there are conditions that permit the expression of this autolytic capacity. Proneness to autolysis seems to be a characteristic not only of certain nutritionally deprived cultures of *S. faecalis*, but also of cells taken from the exponential phase of growth. The exact point at which cells are harvested during exponential growth seems to affect only the rate of autolysis observed. Figure 2 shows the results of an experiment which illustrate the relationship of stage of growth to autolytic capability of *S. faecalis* (54). Cells were harvested at the points of growth indicated by the arrows, washed, and placed in 0.3 M phosphate buffer, *p*H 6.5. The numbers next to the arrows indicate the time in minutes required for these harvested, washed cells to lose 25% of their initial turbidity in the buffer. Most rapid autolysis took place when cells were harvested toward the end of the exponential phase of growth. After the end of exponential growth, the rate of autolysis rapidly decreased to essentially zero. Stationary-phase *S. faecalis* cultures resulting from the deprivation of threonine or valine also failed to exhibit significant autolysis under the same conditions (49).

Several factors seem to be important for autolysis of lysis-prone cells in the exponential phase of growth (49). Among these factors are (i) prevention of continued cell-wall synthesis in either a balanced or unbalanced fashion, (ii) the osmotic environment, (iii) the *p*H of the medium in which lysis is to be observed. The occurrence of autolysis in lysis-prone cells is inhibited by low *p*H. This last factor may help to explain the earlier failure to observe autolysis during, or immediately after, exponential growth

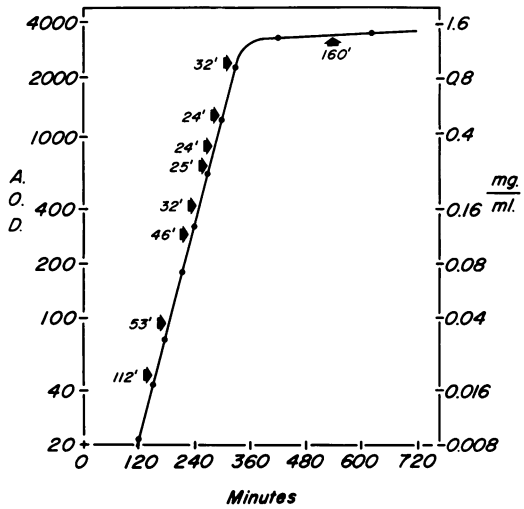

FIG. 2. *Relationship of the growth curve of Streptococcus faecalis 9790 and autolysis as observed in 0.3 M phosphate buffer, pH 6.5 (54). AOD is optical density (× 10^3) adjusted to agree with Beer's law (59). One AOD unit is equivalent to 0.4 μg/ml of dry bacterial substance during the exponential phase. An exponentially growing inoculum was used, resulting in the absence of a lag before the onset of exponential growth. The numbers next to the arrows indicate the time in minutes required for harvested, washed cells to lose 25% of their initial turbidity when incubated in the buffer.*

when the organism was grown in a medium containing low phosphate concentrations (58). There seems to be no specific role for phosphate in the autolytic process, since autolysis has been observed in sodium chloride, sodium acetate, and tris(hydroxymethyl)aminomethane (Tris) buffer (49, 55).

Autolysis and autolytic enzyme systems have been observed in a variety of gram-positive and gram-negative bacteria (20, 33, 34, 63, 64, 65). At least some of these autolytic systems have been found to be most active during or towards the end of the exponential phase of growth when cells are growing and dividing in a balanced fashion (20, 33, 65). The autolytic system found in *S. faecalis* during the exponential phase seems to be highly specific (49). Well-washed walls from cells in the exponential phase will slowly autolyze in phosphate buffer or in distilled water (Fig. 3). This is in contrast to the relative stability of walls from a threonine-deprived culture. Extracts of *S. faecalis* cultures in the exponential phase increased the rate of lysis of *S. faecalis* cells taken from the exponential phase of growth and of isolated cell walls of such cells. These same extracts failed to lyse cells from stationary phase cultures (48, 55). A similar type of specificity has been demonstrated for the autolytic system found in transformable *B. subtilis* cultures (64, 65). Lytic activity of the extracts was nondialyzable and heat-labile (55). The autolytic system does not seem to be a lysozyme. The extracts failed to lyse *Micrococcus lysodeikticus* (48, 55).

It has been proposed by a number of laboratories (33, 51, 63) that these autolytic enzymes play some role in wall growth and cell division. Perhaps existing bonds are broken in the more or less continuous matrix of wall polymers that surround the cell, so that a new bit of completed precursor can be inserted, thereby lengthening the polymer chain. Perhaps the bands of new wall synthesis seen by use of immunofluorescence (6, 7, 8, 9, 10, 28) are initiated by such an autolytic system and correspond to the only areas of wall that are susceptible to the autolytic enzyme system. Such areas may either be absent or modified in walls of older cultures. Several years ago, Mitchell and Moyle (33) observed the presence of hemispherical wall fragments after the action of an autolytic enzyme on *Staphylococcus aureus*.

Thus, the following implications may be made from lytic-enzyme hypothesis. (i) The new bit of wall polymer must be inserted into the existing polymeric chain immediately after the action of the potentially lytic enzyme at that site. Other-

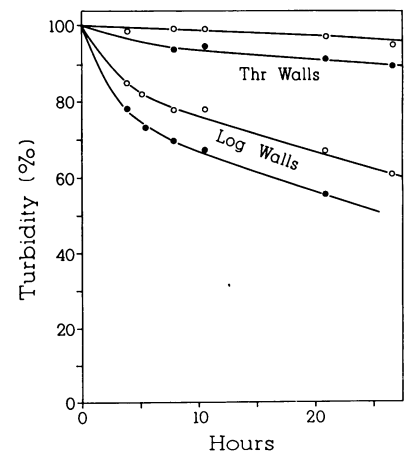

FIG. 3. *Lysis of cell-wall preparations incubated in water (○) or in 0.01 M phosphate buffer, pH 6.5 (●). The upper two lines represent preparations from threonine-deprived cells, and the lower two from exponential-phase cells. The results are expressed as percentages of the turbidity of the incubated tubes at time-zero (49).*

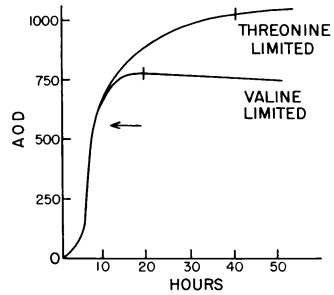

FIG. 4. *Arithmetic plot of the turbidity increase observed under conditions of valine or threonine limitation (61). At the turbidity indicated by the arrow (560 AOD units), the exogenous supply of the single growth-limiting amino acid (valine or threonine) is exhausted. Exponential growth, which occurs up to this point, ceases, and a slow rise in turbidity, at a constantly decreasing rate, occurs. The vertical lines on the curves indicate the point at which cells were harvested for study.*

wise, the enzymatic attack on the wall can result in osmotic fragility. (ii) The susceptible bond attacked by the enzyme occurs only in walls of growing cells and, perhaps, only at those sites of new wall synthesis seen by use of immunofluorescence. It is of some interest to note the following coincidence. The increasing speed of autolysis during exponential growth of *S. faecalis* (Fig. 1) seems to correspond to a decrease in average chain length of the cocci as observed in the Coulter electronic particle counter (60).

Recent experiments indicate that the autolytic system in *S. faecalis* 9790 may be much more complicated than originally anticipated. Discrepancies in the behavior of isolated cell walls, whole cells, and heat-inactivated whole cells, as substrate for lytic extracts of cells in the exponential phase, are currently being investigated.

UNBALANCED WALL SYNTHESIS RESULTING IN WALL THICKENING

A variety of criteria have been used to demonstrate that the increase in turbidity and dry weight of *S. faecalis* cultures resulting from the absence of an exogenous supply of either L-valine or L-threonine is due, in part, to the continued synthesis of cell-wall substance (45, 48, 50, 51, 57). These criteria include: (i) an increase in the cellular content of certain wall-specific components (44) such as rhamnose, amino sugars, and the amino acids, D-alanine, D-glutamic acid, and D-aspartic acid; and (ii) an increase in total wall nitrogen determined after quantitative cell disruption. Figure 4 shows an arithmetic plot of growth of *S. faecalis* under conditions of valine or threonine limitation. At the point indicated by the arrow, the available supply of either valine or threonine is exhausted from the growth medium. The observed increases in turbidity were shown to be accompanied by a nearly corresponding increase in cellular dry weight (45, 51). Exhaustion of valine from the culture medium resulted in an increase in cellular dry mass of approximately 40% in about 10 hr, and then leveled off. Threonine exhaustion resulted in a rise in cellular dry weight that continued for a much longer period of time. After 35 to 40 hr, threonine-deprived cultures reached levels which were 80 to 100% greater than at the time of threonine exhaustion. Valine-deprived (17 hr) and threonine-deprived (41 hr) cultures in the exponential phase were disrupted by means of shaking with Ballotini glass beads (45, 51). The time courses of such disruptions (Fig. 5) were followed by determining nitrogen in the soluble and insoluble fractions. The percentage of total cellular nitrogen which remained insoluble after complete disruption increased from 11% in exponential phase cells to 22% after valine deprival and 28% after threonine deprival. From such data it was estimated that the synthesis of additional cell-wall substance was responsible for about 70% of the observed gain in cellular dry weight in both these instances (45, 51).

The magnitudes of these increments are summarized in Table 1 and indicate that the amount of cell-wall substance per culture approximately doubles after valine exhaustion and approximately triples after threonine exhaustion. A

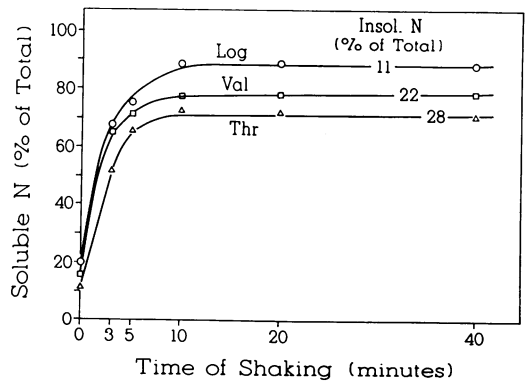

FIG. 5. *Time-course of mechanical disruption of exponential-phase, and valine- and threonine-deprived, cells (51). Disruption was followed by determining nitrogen on the soluble and insoluble fractions at the indicated time intervals.*

TABLE 1. *Cell-wall substance in exponential-phase, threonine- and valine-deprived cultures*

Culture	Cell substance	Cell wall (mg)		
		Per 100 mg of cells	Per culture	Net gain
	mg			
Exponential phase	100	25	25	
Valine-deprived (17-hr incubation*)	140	38	53	28
Threonine-deprived (41-hr incubation*)	180	44	79	54

* Time is from an exponentially growing inoculum of 0.4 μg/ml (dry weight).

concomitant net gain in cellular protein was not observed in the absence of either of these nutritionally essential amino acids. This is in agreement with current concepts of protein synthesis (14), which indicate that all of the amino acids that are components of a protein must be present simultaneously for protein synthesis to occur. There are examples of proteins, some of bacterial origin, that lack one or more of the common amino acids (14). However, these proteins seem to be more the exception than the rule. Amino acid analyses of the soluble cytoplasmic fraction of *S. faecalis* 9790 indicate that all of the common amino acids are present (57). This does not eliminate the synthesis of proteins lacking valine or threonine (undoubtedly a small proportion of the total cellular protein), nor does it mean that there is no turnover or change in protein makeup after valine or threonine depletion. In fact, evidence has been obtained which indicates that breakdown and resynthesis of new protein may occur (62).

Evidence also has been obtained for the continued synthesis of additional cell-wall substance in a medium grossly deficient for this organism (50). This "wall medium" lacks nearly all of the essential and nonessential amino acids and vitamins. It contains glucose, sodium acetate, phosphate buffer, inorganic salts, and only the following amino acids: cysteine, lysine, alanine, glutamic and aspartic acids. Except for cystine, all of these amino acids are important components of the cell wall of this organism. An approximate doubling in both cellular rhamnose content and insoluble (cell-wall) nitrogen after 22 hr of incubation in this "wall medium" were taken as indications of continued synthesis of cell-wall substance. In these experiments, a net loss rather than a gain of non-cell-wall (soluble fraction) nitrogen was observed. This again was taken as an indication of the absence of the net synthesis of cellular proteins.

Additional evidence for the occurrence of cell-wall synthesis and the absence of net protein synthesis in this "wall medium" was obtained by use of inhibitors. The addition of penicillin at concentrations lower than that required to inhibit growth of the same number of organisms resulted in lysis of the cultures (46). Cycloserine addition could also result in lysis, but the inhibitory effect of cycloserine was reversed competitively by D-alanine. Penicillin inhibition and the resultant lysis in the absence of balanced growth was somewhat unexpected in view of the evidence obtained with other bacteria that growth and, perhaps, protein synthesis are required for penicillin action (5, 11, 23, 29). It is interesting to note that the addition of chloramphenicol to this system affected neither the overall turbidity increase observed, nor the lytic action of penicillin. These results indicate that, for this organism, under these conditions, balanced growth and protein synthesis is not a requirement for penicillin action, and that chloramphenicol-sensitive protein synthesis is not a major factor in the observed increases in turbidity. The only requirement for penicillin action under these conditions seems to be the occurrence of active cell-wall synthesis.

A number of observations from other laboratories indicate that the synthesis of cell-wall polymers can be dissociated from protein synthesis. For example, Hancock and Park (15) and Mandelstam and Rogers (24) showed that mucopeptide synthesis can occur in *S. aureus* in the presence of growth-inhibiting concentrations of chloramphenicol. Chatterjee and Park (5) showed that chloramphenicol does not inhibit mucopeptide synthesis in a cell-free system derived from *S. aureus*. Also, Hash et al. (17) presented evidence that, similar to chloramphenicol inhibition, tetracycline treatment also results in inhibition of protein synthesis with the continued synthesis of cell-wall substance in *S. aureus*.

In the absence of direct morphological observations, the interpretation of the synthesis of additional cell-wall substance in terms of individual cells is somewhat complex. There appear to be several alternative mechanisms to explain the fate of the additional cell-wall substance synthesized. The presence of more wall could result in (i) a thicker wall structure, (ii) a more dense wall structure, or (iii) the same thickness or density of substance enclosing the protoplasm in a way that results in a greater surface area per culture. Examples of the third alternative in-

clude: (i) the same number of larger cells containing the same dry weight of protoplasm in a more "dilute" form, that is, containing more water; (ii) additional divisions could occur resulting in a larger number of cells of approximately the same size; (iii) the shape of the cells could deviate further from spherical. (A perfect sphere has the lowest ratio of surface area to volume.)

Phase-contrast light microscopy of whole cells did not answer the question. Cell counts, either viable or total, are complicated in interpretation by the chain-forming nature of the organism. The Coulter electronic particle counter did not discriminate between chains of two or more cocci and larger individual cells (60). In bacterial cultures, a continuous spectrum of cell volumes has been observed repeatedly (1, 21, 22).

In an attempt to gain information concerning the three alternatives, studies were initiated in collaboration with Manfred E. Bayer at the Institute for Cancer Research. An electron microscopic technique, especially developed for this problem, was applied (3, 48, and *unpublished data*).

A drop of washed cell suspension was placed on a carbon grid to which was added approximately an equal volume of 2% unbuffered osmium tetroxide. After a suitable interval (about 15 to 20 sec for log-phase cells, 30 to 60 sec for the other cell types), the excess solution was removed with filter paper, and the specimen was immediately dried and examined in a Siemens Elmiskop I electron microscope at direct magnifications of 20,000 to 40,000 diameters. Figure 6 shows electron micrographs of portions of cells taken from (i) the exponential phase of growth, (ii) a valine-deprived culture, and (iii) a threonine-deprived culture. With this staining technique, very little of the osmium seems to remain in the area occupied by the cell wall. The wall is seen as a rather bright layer between two areas of high electron contrast. The latter corresponds to the strongly stained protoplast inside the cell wall and to the outer zone of osmium compounds gathered around the bacterial cell. There are some irregularities in the thickness of the walls as seen on individual cells and from cell to cell within a single cell type. Some of this can be attributed to the method of preparation. Flattening of the specimen by the forces of surface tension sometimes causes the walls to appear thicker. When an extremely massive external layer of osmium tends to cover the wall, it appears thinner. Absence of negative stain leaves the outer margin of the cell wall nearly invisible. However, these variations in thickness of walls on individual cells and from cell to cell within a single cell type are relatively small in comparison with the differences in thickness between walls of exponential-phase cells and of the two nutritionally deprived cell types. A large number of cells of all three types have been observed, and the cell walls of both valine- and threonine-deprived cells appear to be two to three times as thick as those of the parent exponential-phase cells. The walls of the two amino acid-deprived cell types appear to be of about equal thickness despite the fact that threonine deprivation leads to a much larger gain in cell wall than does valine deprivation (Table 1). Perhaps the difference in wall thickness between valine- and threonine-deprived cells is too small to be detected by this method. Alternatively, one or more of the considerations mentioned above, such as a difference in shape, size, or number of cell units present, may result in walls of nearly equal thickness.

These electron microscopic observations are stated in qualitative terms. Quantitative interpretation is complicated by (i) problems which are part of the experimental method employed, such as shrinkage and distortion in the drying process; (ii) possible differences in number of individual cell units present; and (iii) the impossibility of using a simple geometric description for the shape, volume, and surface area of these cell units. Large differences in the average diameter between the different cell types were not observed. However, small differences in diameter, as well as a change in shape, can result in a considerable change in volume and surface area.

In the "wall medium," deprivation of nearly all amino acids, except those that are components of the cell wall of *S. faecalis*, results in synthesis of additional cell-wall substance (50). It is likely that these conditions also result in a thickened wall structure, although this has not been demonstrated.

As observed by this negative staining technique, cell walls of valine- or threonine-deprived cells appear to be approximately 250 to 300 A in thickness, compared with an average wall thickness of about 100 A for cells in the exponential phase. These values are within the range of thickness reported for cell walls of a number of bacterial species observed by use of other electron microscopic techniques (44).

As mentioned above, conditions other than amino acid starvation can result in inhibition of protein synthesis and the continued synthesis of cell-wall mucopeptide. Thus, it became of interest to see whether cell-wall thickening could be demonstrated after chloramphenicol inhibition. The addition of chloramphenicol to a culture of *S. faecalis* growing exponentially in a

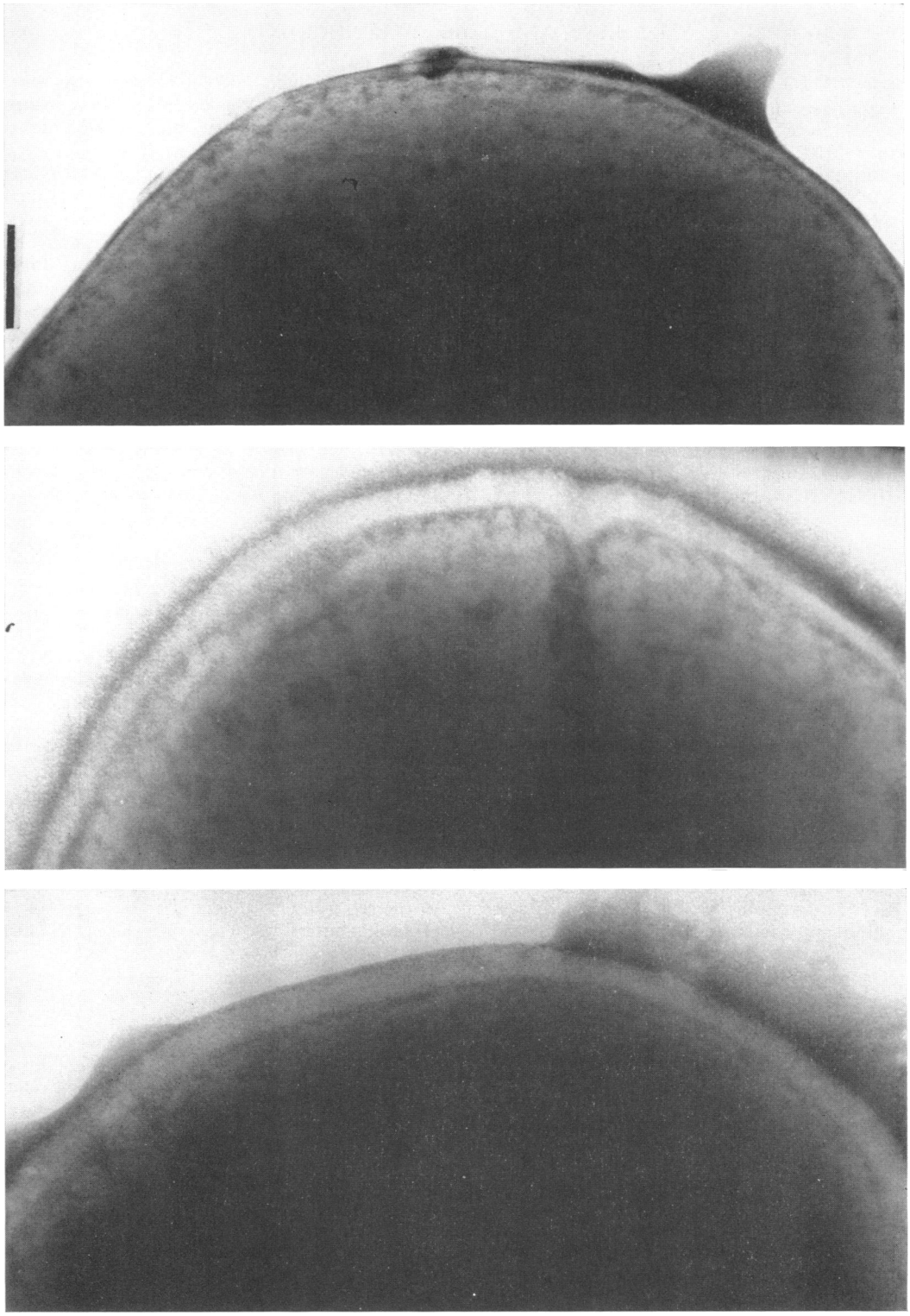

FIG. 6. *Portions of electron micrographs of Streptococcus faecalis cells. (top) Cells taken from the exponential-growth phase. (center) Cells from a valine-deprived culture (17-hr incubation). (bottom) Cells from a threonine-deprived culture after 40 hr of incubation (3, 48). Whole cells were fixed and negatively stained with osmium. For details of the method, see text. The bar on the figure indicates 0.1 μ. In collaboration with M. E. Bayer.*

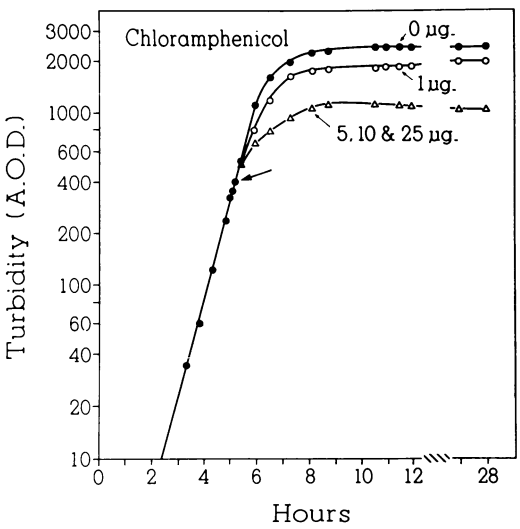

FIG. 7. *Effect of chloramphenicol on exponentially growing cultures of Streptococcus faecalis. Turbidity is expressed in AOD units. Chloramphenicol, at the concentrations indicated, was added at AOD 400.*

complete medium closely resembles the effect of threonine exhaustion (Fig. 7). Upon the addition of chloramphenicol, exponential growth ceases, and a slow rise in turbidity ensues. With the same negative staining technique, samples of *S. faecalis* were examined in the electron microscope before and after the addition of 15 µg/ml of chloramphenicol to an exponentially growing culture (Bayer and Shockman, *unpublished data*). The results are shown in Fig. 8. Cell-wall thickening after chloramphenicol treatment seems to be of about the same magnitude as that seen after valine or threonine deprival.

These results indicate that, when protein synthesis in *S. faecalis* is inhibited either by withdrawal of an essential protein precursor, such as valine or threonine, or by the addition of an inhibitor of protein synthesis, such as chloramphenicol, continued cell-wall synthesis occurs. This results in cells with cell walls that are two to three times thicker than those of the exponentially growing parent culture.

Comparisons of thickness of bacterial cell walls grown under different conditions have been made in a few other instances. Hash et al. (17) studied the effects of tetracycline on a culture of *S. aureus* H nearing the end of the exponential growth phase in a complex medium. Replotting their absorbancy measurements on an exponential scale [after adjustment of their values for deviation from Beer's law for particles in suspension (19, 47, 59) to make them approximately proportional to cellular mass] reveals that the turbidity change produced by tetracycline inhibition of *S. aureus* H closely resembles that for chloramphenicol inhibition of *S. faecalis* shown in Fig. 7. Tetracycline inhibition resulted in a nearly immediate cessation of exponential growth and a somewhat larger rise in turbidity than that observed for *S. faecalis* (Fig. 7). On the basis of a series of biochemical experiments, Hash et al. (17) concluded that exposure of *S. aureus* H to tetracyclines resulted in an immediate block at some stage of protein synthesis. Tetracycline-inhibited cells continued to synthesize nucleic acids and cell-wall mucopeptide. Hash and Davies (16) presented electron micrographs of sectioned *S. aureus* H cells which showed thicker cell walls resulting from exposure to tetracycline. Thus, the effect of another inhibitor of protein synthesis on a different organism is quite similar to those observed here with *S. faecalis*.

Holden (18) has shown that vitamin B_6-deficient cells of *Lactobacillus arabinosus* contain only about one-half the amount of cell-wall substance when compared with normal B_6-sufficient cells. These cells have a thinner-than-normal cell-wall structure (18). Supplying vitamin B_6-deficient cells with the vitamin results in synthesis of the wall structure and in the cells regaining certain of their physiological properties. Murray et al. (35) recorded a thinning of *S. aureus* walls during penicillin treatment in an osmotically protective medium. This was attributed to the inhibition of cell-wall synthesis and continued cytoplasmic synthesis resulting in an increase in diameter of the cells without concomitant cell-wall growth. Cole (9, 10), using the immunofluorescent technique, examined the effect of chloramphenicol on wall replication in *S. typhosa*. In the absence of chloramphenicol, wall replication in this organism was observed to occur by means of diffuse intercalation of new wall material among the old. Chloramphenicol treatments resulted in large nondividing cells that showed definite nonfluorescent irregular interruptions between fluorescent areas of the wall marker. These results were interpreted as a visual demonstration of continued wall synthesis in the presence of chloramphenicol. The pictures obtained by Cole are compatible with (and could be a reflection of) wall thickening resulting from continued cell-wall synthesis.

A completely hypothetical scheme (Fig. 9) might help to explain our findings on cell-wall thickening of *S. faecalis*, as well as those of Cole

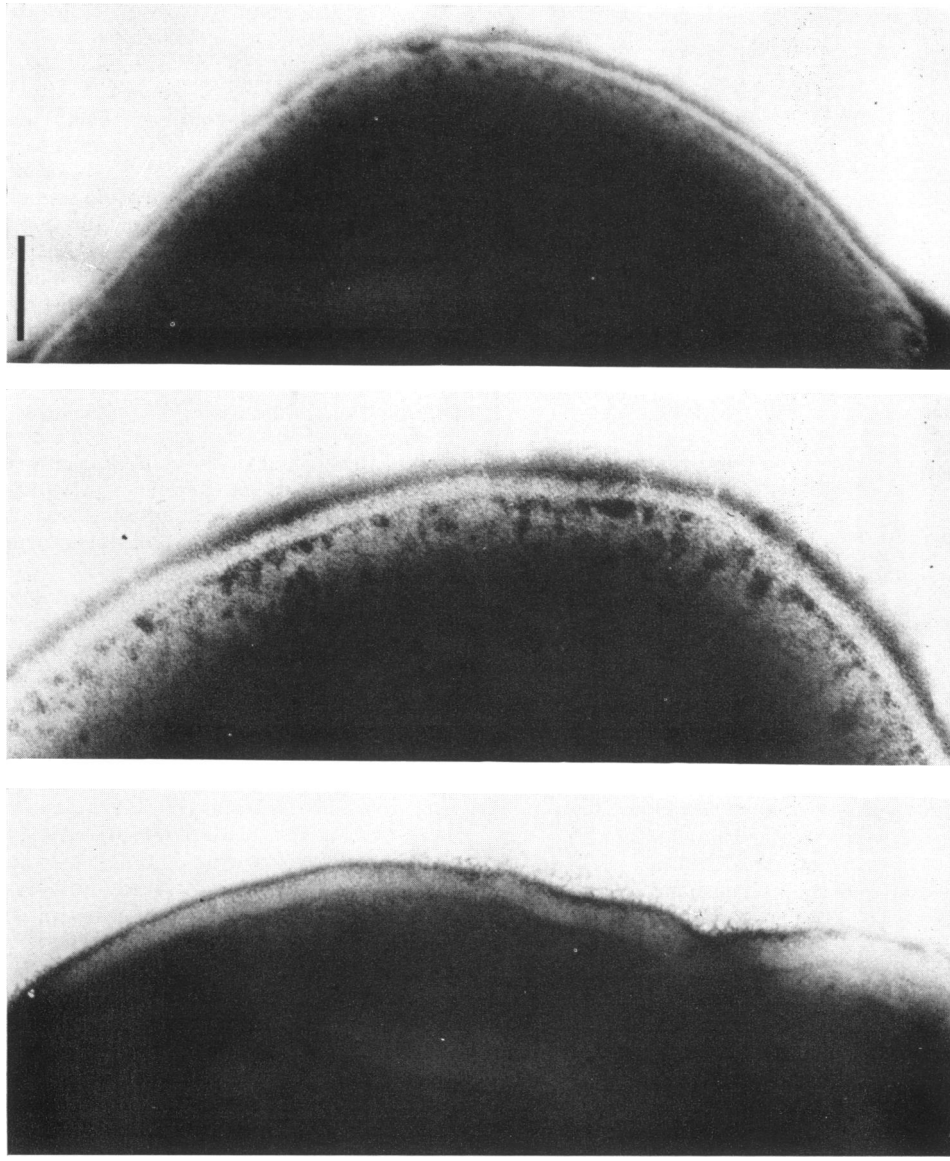

FIG. 8. *Portions of electron micrographs of Streptococcus faecalis cells. (top) Cells taken from the exponential-growth phase. (center) Cells 1 hr after the addition of 15 μg/ml of chloramphenicol. (bottom) After about 17 hr of treatment with the same concentration of the antibiotic. Cells fixed and stained by the same method used for the preparations shown in Fig. 6. The bar on the figure indicates 0.1 μ.*

with chloramphenicol-inhibited *S. typhosa*, in terms of the results obtained with fluorescent-antibody labeling of bacteria during balanced growth (6, 7, 8, 9, 10, 28). Figure 9A is a diagrammatic representation of balanced cell-wall replication and cell division that has been observed to occur in *S. pyogenes* by use of the fluorescent-antibody technique (10). A similar, but perhaps slightly different, mode of wall replication has been observed recently in a strain of *S. faecalis* (7). Cell-wall synthesis is initiated at the coccal equator (band 1). Before the completion of cell division, a second band of wall synthesis (bands 2) is initiated at two bands around the coccus, midway between the initial band of wall synthesis and the ends of the coccus. After

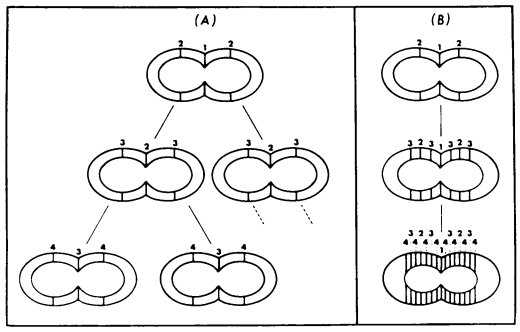

FIG. 9. *Hypothetical scheme to explain cell-wall thickening on the basis of the initiation of multiple sites of cell-wall synthesis. Each figure is intended to represent a cross section of a cell unit. (A) Schematic representation of cell division as observed with Streptococcus pyogenes by use of the immunofluorescence technique (10). (B) Hypothetical representation of unbalanced synthesis of cell-wall substance, resulting in wall thickening. The numbers indicate the sequence of the initiation of new rings or bands of wall synthesis.*

division at the first band, the third set of bands (bands 3) of wall synthesis are initiated, with one per coccus. The hypothetical scheme for unbalanced wall synthesis in the absence of net protein and general cytoplasmic synthesis is illustrated in Fig. 9B. In this case, the bands of wall synthesis are initiated just as they are in the balanced picture on the left. The second set of bands (bands 2) is initiated before the first has been completed midway between the first band and the end of the coccus. However, in this instance, net protein synthesis and cytoplasmic synthesis have been inhibited and are not keeping up with the continued synthesis of the wall polymers at the new sites of wall synthesis. An increase in cell size and completion of cell division is, therefore, either disturbed or completely inhibited. The currently available data concerning cell size and numbers for such conditions are difficult to interpret. Therefore, we do not know whether any further cell divisions occur. Continued initiation of new bands of wall synthesis (bands 3 and 4, Fig. 9B), midway between the previously initiated bands of wall synthesis, eventually saturates all of the potential sites available. The new wall polymers made at these sites could then result in thickening of the cell walls of individual cells. If the initiation of new bands of wall synthesis occurs by means of controlled wall hydrolysis (e.g., an autolytic system), inhibition of wall synthesis (e.g., absence of required precursor or addition of an inhibitor) could result in cell lysis.

S. faecalis cells with thickened walls resulting from amino acid deprivation differ in a variety of properties from the thinner-walled exponential-phase cells (48, 49). In general, they are more resistant to a number of deleterious agents including the *S. faecalis* wall lytic-enzyme system (49). A small, but perhaps significant, quantitative difference in composition of the two types of walls has been observed. The amounts of each of the five principal amino acids found in the wall of this strain average 18% higher in walls from threonine-deprived cultures than in walls from exponential-phase cells (57). This could indicate either more mucopeptide or more extensive cross-linking within the wall mucopeptide. These differences in biological properties and chemical composition would be consistent with the idea that the cell-wall synthesis which occurs while protein synthesis is inhibited somehow differs from that which occurs during "balanced" growth. Some of the more recently accumulated data on the structure and synthesis of bacterial cell walls suggest that this might occur at the latter stages of wall synthesis (27, 40, 42).

The fact that bacterial cells continue to increase in mass and divide at an exponential rate for as long as an adequate nutritional and physical environment is provided would indicate that, during the exponential growth phase, cell-wall synthesis must somehow be integrated with the other cellular synthetic processes. Preliminary evidence (G. Toennies, *personal communication*) indicates that the ratio of cell-wall substance to total cell substance present during exponential growth is relatively constant over a threefold range of growth rate. It is only after the net synthesis of protein is inhibited that synthesis of the cell-wall polymers exhibits ". . . an alarming degree of independence from other biosynthetic processes" (36). Somewhat similar phenomena have been observed for the biosynthesis of other cellular macromolecular components. For example, "relaxed control" strains of bacteria continue to synthesize RNA when deprived of one or more amino acids (37, 53). In addition, there are a number of observations (4) which indicate that chloramphenicol inhibition of a variety of different bacterial species results in an accumulation of RNA.

Previously, a hypothesis was presented (51) suggesting that, during exponential growth, cell-wall synthesis may normally lag slightly behind protoplast synthesis. Perhaps some of our results concerning both unbalanced cell-wall thickening and the autolytic properties of exponential-phase cells will become interpretable in terms of this hypothesis. The biochemical

structure and the fate of cell walls synthesized during the inhibition of protein synthesis, and the nature of the autolytic enzyme system and its substrate, are currently being investigated.

SUMMARY

Biochemical and morphological studies have indicated that unbalanced growth of *S. faecalis* (9790) can result in wall thickening or autolysis. Inhibition of protein synthesis can result in wall thickening, and inhibition of wall synthesis can result in autolysis. There are quantitative and perhaps qualitative differences between specific nutritional and inhibitory situations that result in the same general effect (48, 61). These differences, which may have considerable significance, have not been emphasized in this paper. Other cellular components, such as DNA, RNA, ribosomes, protein, and membrane, are affected also by a change in environment (36, 61, 62). Our present state of knowledge allows us only to speculate about the biochemical mechanisms that regulate either balanced growth and cell division or the more specialized unbalanced states. The hypothetical scheme presented in Fig. 9 is an attempt to bring together the somewhat diverse observations on cell-wall thickening and autolysis with those on cell-wall replication as observed by use of immunofluorescence. Perhaps these speculations can eventually lead via experimental findings to knowledge of the true situation.

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